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RESEARCH ARTICLE

Albumin-Suppressed Haemoglobin Capture in Iron-Porphyrin Molecularly Imprinted Hydrogels

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Abstract
The artificial receptors functionality is feasible only if the target signal stays independent from other protein sorption processes. The current study is focused on determining the possibility to use the chemistry of the iron-porphyrin complex as a tool for turning haemoglobin-imprinted hydrogel into the protein-receiver with albumin suppressing abilities. Comparison is done according to such criteria as the amount of proteins captured by imprinted and non-imprinted hydrogels without an auxiliary co-monomer, free-base porphyrin and iron-porphyrin. These proteins include bovine haemoglobin and bovine serum albumin. As the main parameters of receptor functionality, the four following factors were considered: high target retention, minimal interference adsorption, the best possible selectivity of the MIP and the highest apparent affinity constant. The only composition meeting the criteria was iron-porphyrin, which demonstrated high amounts of haemoglobin retention (81.2 %), low albumin binding rate (18.5 %), high selectivity (4.40) and appropriate apparent dissociation constant (3.40×10^{-7} M). While the second sample had higher values regarding the amount of haemoglobin captured, it still managed to adsorb a lot of albumin (35.7 %) and achieved selectivity value of 2.17. In its turn, non-modified sample failed to create the required window of difference between proteins – 36.5 percentage points. Thus, the research shows the affirmative answer: yes, the iron centre is changing the function of the receiver due to its ability to retain haemoglobin while suppressing albumin adsorption at the same time.

Keywords: protein imprinting; hydrogel receptor; haemoglobin; bovine serum albumin; metalloporphyrin; interference rejection; analytical sensing

1. Introduction

The capture of proteins specifically remains an issue in analytical chemistry as there are plenty of macromolecules found in natural samples that are capable of interaction with any given surface. Naturally occurring recognition elements may be sufficiently selective for most uses, but the latter usually imply that they need to be stored in a special way and are likely to degrade, not to mention the relative high cost associated with reproduction on a large scale. As a result, synthetic recognition components have become increasingly important for developing reliable techniques in sample preparation and sensing, with molecular imprints standing out as the key examples. Molecular imprints refer to polymerisable groups organized in respect to the structure of a template and captured in a network structure retaining template's recognition properties once removed [1,2].

The process of imprinting proteins proves to be particularly difficult compared to the synthesis of molecular imprints for small molecules. Proteins are quite large in size, hydrated, capable of assuming various conformations, and contain a wide variety of chemical groups. Their exposed surfaces are composed of charged moieties, hydrophobic groups, hydrogen donors and acceptors, and metal-binding groups as well. While a perfectly good approach for imprinting of small molecules, it is doomed to failure when it comes to protein imprinting because the latter can either produce insufficiently flexible material, deeply hidden binding region, and/or excessive nonspecific interactions. Review articles in the field of macromolecular imprinting stress the importance of shape complementarity, surface properties, accessibility, and use of aqueous solvent [3–5].

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One way to address the problems mentioned above is to apply hydrogels to protein imprinting. Unlike highly crosslinked organic networks, hydrogels remain flexible and retain protein hydration. Additionally, hydrogel sheets make possible proximity of the binding region to the surface. While several studies confirmed that hydrogel imprinting indeed yields specific binding sites, further studies demonstrated that the polymerization procedure, polymer composition, swelling properties, post-imprinting modifications, and protein orientation affected binding constants and selectivity [6–8]. When speaking of the analytical quality of a molecular imprint, the protein binding property itself is less important than its ability to distinguish between specific and nonspecific bindings.

The target protein selected for the purpose of metal-assisted imprinting is bovine haemoglobin because of its characteristic surface chemical groups, i.e., haem. As for the interfering protein, bovine serum albumin is chosen because of its ability to attach easily to different types of surfaces, including polymeric ones. At that, both proteins need to show identical affinity for the receptor in order for the latter to be considered inefficient analytically. On the contrary, an analytical receptor is expected to bind strongly with the target and weakly with the interferer. In this case, it needs to have a high target-to-interference ratio and decent affinity within measurement time.

Metallic complexes of porphyrins are a particularly interesting tool here as they introduce additional specificity in recognition by introducing specific chemically defined groups into the hydrogel. Along with macroaromatic ring system of the ligand, free-base porphyrin provides additional contact through hydrogen bonding. Metalloporphyrins also add the possibility to use the central metal atom to form coordination bond with the surface through an electron-donating group. Indeed, porphyrin and metalloporphyrin motifs were used successfully before as recognition centers [9,10]. Even today, molecularly imprinted polymers are recognized as synthetic recognition layers [11]. Still, the question is if the metallic complex of a porphyrin motif improves its selectivity for a target protein.

The modern advances in diagnostics and biosensing indicate that the current need for this kind of approach. Molecular imprints are viewed as antibody mimics for use in biofluids and point-of-care testing, but such use suffers from several problems related to the matrix complexity, protein corona, fouling, and inability to regenerate the recognition layer without damaging its quality [12–14]. Therefore, the analytical recognition layer needs to be evaluated considering its ability to separate specific and nonspecific binding. This cannot be done based solely on imprinting factor, selectivity, and/or dissociation constant values individually.

Therefore, the research problem that this paper addresses is the following one: *can the complex of iron with the porphyrin group help create a hydrogel imprint with haemoglobin binding specificity against the bovine albumin interference?* This analysis is centered around a two-probe test series of an analytical recognition layer and its ability to discriminate between target and interfering protein using interfering-gate parameters. In other words, no score evaluation will be provided; only adequate molecular combination will be found.

2. Materials and analytical methodology

2.1. Measurement set and receptor chemistries

The selectivity, affinity, and percentage binding measurements of hydrogel molecular imprints modified with porphyrins and iron-porphyrins reported by Sullivan et al. [15] will be used as the receptor measurement set to evaluate its discrimination capabilities analytically. There are three hydrogel chemistries in total: a molecular imprint of bovine haemoglobin, a molecular imprint of bovine haemoglobin modified by free-base porphyrin, and a molecular imprint of bovine haemoglobin modified by iron-porphyrin. For each chemistry, non-imprinted analogue is provided.

The measurements considered include MIP/BHb, NIP/BHb, MIP/BSA, NIP/BSA, imprint factor, MIP selectivity factor, NIP selectivity factor, and apparent dissociation constants. These measurements combine into the following parameters: target-interference window, imprint gain, albumin rejection against non-imprinted analogue, selectivity amplification against non-imprinted analogue, and affinity discrimination between MIP and NIP hydrogel sheets.

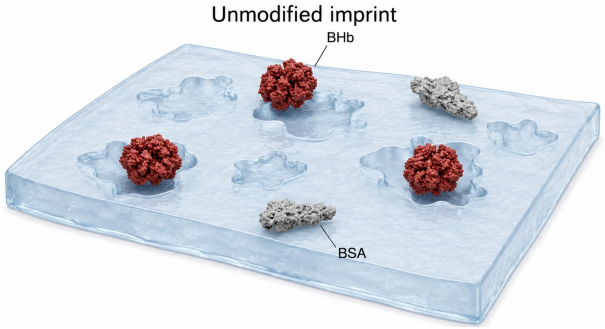

Figure 1: Reference imprint.

The unmodified hydrogel represents a control hydrated cavity. This is important because its impact needs to be compared against the effect of the porphyrins and iron porphyrin. The presence of binding sites shaped like the template is what makes this material special. The figure below provides a depiction of this material under standard conditions, where the decision on the effect of the macrocycle must be made.

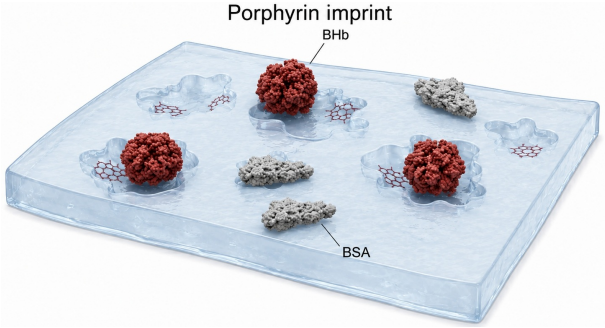

Figure 2: Porphyrin imprint.

The concept of free base porphyrin exploits the mechanism of macrocyclic chelation but lacks a metal ion. This is significant because although aromatics and hydrogen bonding could help with the binding to haemoglobin, they may not suffice to prevent albumin binding. This is demonstrated by the analysis of the binding process done later. However, the presence of a metal in the core of metalloporphyrin suggests

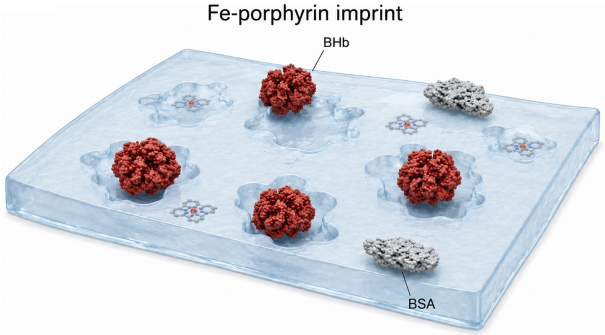

Figure 3: Iron-porphyrin imprint.

that there is a possibility for establishing a coordinating interaction. That would lead to creation of a gap between the two interactions, thereby ensuring specificity.

2.2. Interference-gated evaluation

The current analysis has been developed with respect to adequacy of the measures rather than maximal numbers. In particular, four gates have been set to ensure the quality of material: strong haemoglobin retention by imprinted gel (first gate), reduced albumin binding by imprinted gel (second gate), selectivity factor higher than three (third gate), and apparent dissociation constant not larger than 5.0×10^{-7} M (fourth gate).

As in the case of criteria, gate settings are based on comparative standards rather than universal regulations. At least 75 % haemoglobin retention allows for identifying materials that retain the target proteins efficiently. On the contrary, no more than 25 % albumin binding helps identify materials with less affinity to interfering proteins. The selectivity factor of at least 3 helps determine target preferability to albumin, while apparent dissociation constant of up to 5.0×10^{-7} M proves high target binding capacity.

Interference-gated receptor adequacy

Criterion	None	Porphyrin	Fe-porphyrin
BHb retention >=75%	Fail	**Pass**	**Pass**
BSA uptake <=25%	Fail	Fail	**Pass**
MIP selectivity >=3	Fail	Fail	**Pass**
MIP Kd <=5e-7 M	Fail	Fail	**Pass**

Figure 4: Adequacy gates.

The logic underlying the decision is transparent from the analysis. In particular, non-imprinted material does not pass any of the four gates since the haemoglobin retention is below the threshold, the albumin binding affinity is high, and the selectivity factor is less than 3. Porphyrin imprint passes target-retention gate, but fails remaining gates. The most successful material is iron-porphyrin imprint since it passes all four gates.

3. Results and discussion

3.1. Target capture and albumin interference

Table 1 shows protein binding measures acquired in the experiment. That helps establish a comparison base for measuring interference and target properties of the material. The imprinting hydrogel captures haemoglobin and albumin at 73.8 % and 37.3 % levels. Free base porphyrin increases haemoglobin affinity up to 77.4 % but increases albumin affinity to 35.7 %. Iron-porphyrin imprint provides the highest possible difference between the target and interference, retaining haemoglobin at 81.2 % with no more than 18.5 % albumin affinity.

Table 1: Protein-binding percentages for the receptor chemistries.

Co-monomer	Protein bound (%)			
	MIP-BHb	NIP-BHb	MIP-BSA	NIP-BSA
None	73.8±0.2	50.4±0.7	37.3±0.3	50.4±0.1
Porphyrin	77.4±0.3	46.1±1.3	35.7±0.2	43.5±0.3
Fe-porphyrin	81.2±0.4	47.0±0.3	18.5±0.1	46.5±0.1

The comparison of binding affinities demonstrates that the main benefit of iron-porphyrin imprint does not relate to higher haemoglobin affinity, as it only increased 3.8 %. The important part here is the decrease in albumin affinity down to 18.5 % compared to 35.7 %. Therefore, addition of a metal center to the porphyrin structure improves albumin affinity.

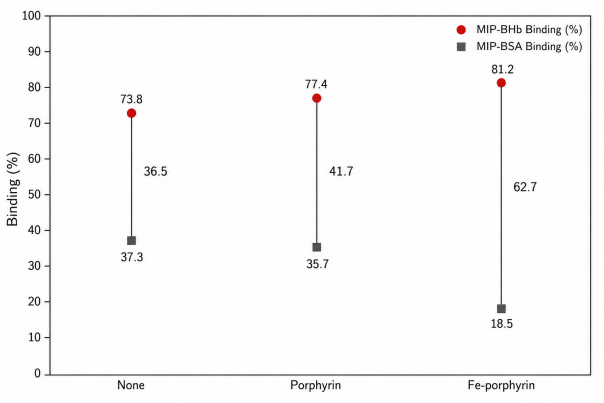

Figure 5: Protein binding.

It becomes obvious from the graph showing both target and interference binding capacity that the most notable difference occurs with iron-porphyrin imprint. Although there is little difference in binding between haemoglobin and albumin with non-functionalized and free base porphyrin, it becomes much wider with iron porphyrin imprint. The reason is that a sensor's signal depends upon both good and bad target capture.

3.2. Derived target–interference window

The target interference window can be calculated as the difference between percent of target (haemoglobin) and interference (albumin) binding to the material. The calculated windows amount to 36.5 %points for the unmodified MIP, 41.7 %points for the porphyrin MIP, and 62.7 %points for the iron porphyrin MIP.

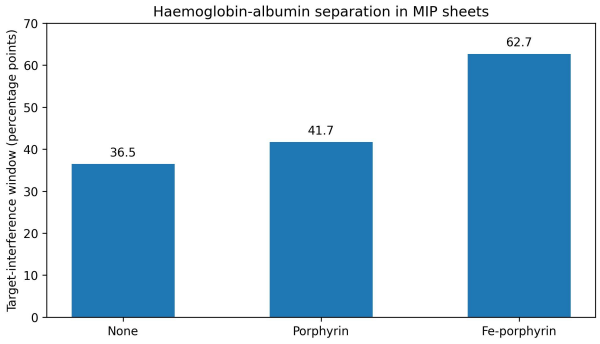

Figure 6: Target window.

The plot of target window allows explaining why iron-porphyrin complex cannot be considered just a better binder. Increased affinity to proteins should mean that the hydrogel binds more haemoglobin and albumin, but iron-porphyrin imprint provides the widest window since it retains almost all haemoglobin but rejects almost all albumin.

3.3. Imprinting contrast and selectivity amplification

The graph of target window shows how non-imprinted imprint creates the largest window. As stated above, increased affinity to proteins leads to increased affinity to both target and interfering species. However,

the imprint generated using the iron porphyrin increases selectivity of target vs interference binding.

Table 2: Imprinting and selectivity factors.

Co-monomer	Imprinting factor	MIP selectivity factor	NIP selectivity factor
None	1.46	1.98	1.00
Porphyrin	1.67	2.17	1.06
Fe-porphyrin	1.73	4.40	1.01

Selectivity and imprinting are based on two different recognition principles: imprinting compares imprinted with non-imprinted gels, whereas selectivity refers to MIP target affinity and NIP protein affinity. While iron-porphyrin hydrogel does not demonstrate superiority in terms of imprinting factor, its unique advantage is selectivity factor. Thus, iron-porphyrin material demonstrates the best discrimination between haemoglobin and albumin.

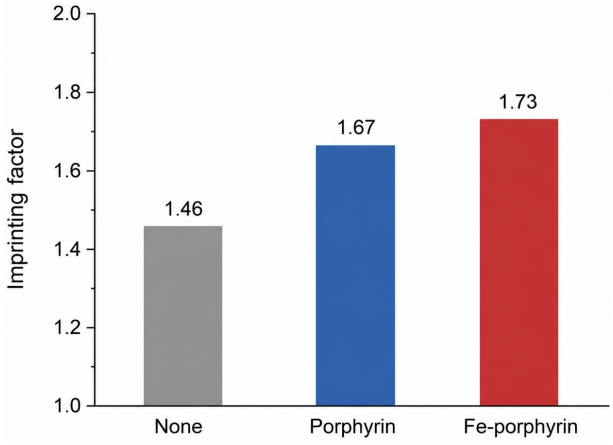

Figure 7: Imprinting factor.

As it is evident from the graph showing imprinting factor values, porphyrin alteration does not make iron porphyrin special. Although it slightly improves imprinting factor (by 17 %), this effect is not sufficient to demonstrate the material's superiority. Therefore, it cannot be said that addition of porphyrin group into non-imprinted hydrogel results in effective recognition.

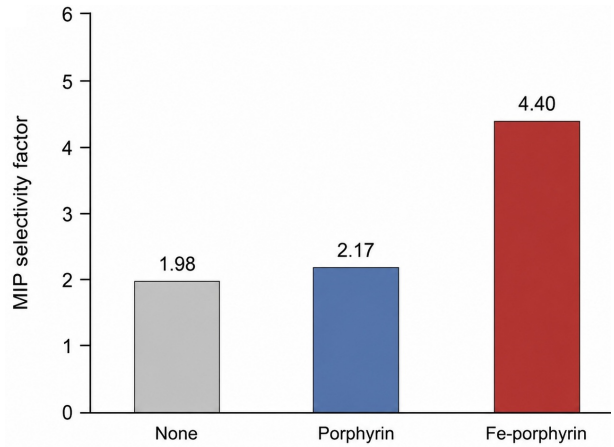

Figure 8: MIP selectivity.

On the contrary, the selectivity factor of the material with target provides better evidence of effectiveness. In particular, free base porphyrin provides only marginal benefits, whereas iron-porphyrin imprint increases selectivity of haemoglobin more than double compared to the non-imprinted polymer. Thus, iron-porphyrin hydrogel is unique because of its selectivity to haemoglobin.

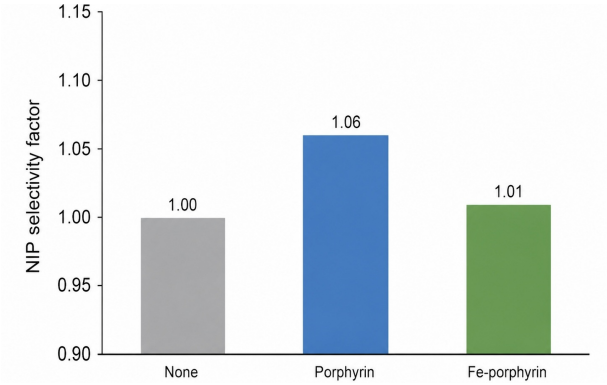

Figure 9: NIP selectivity.

The selectivity of the NIP plot does not allow overrating the effect produced by porphyrin moiety alone. In particular, values close to 1 suggest that the non-imprinted polymer with porphyrin and/or iron-porphyrin alteration cannot discriminate haemoglobin from albumin. However, selectivity of target imprint appears in such material, as the template is used to create a suitable binding site.

3.4. Apparent affinity and signal durability

The dissociation constants calculated based on apparent affinity are presented in Table 3. It should be noticed that, while the apparent dissociation constant for MIP amounts to 10.13×10^{-7} M in the case of non-modified hydrogel, for porphyrin hydrogel, it lowers to 5.30×10^{-7} M, whereas for

Table 3: Apparent dissociation constants.

Co-monomer	MIP apparent dissociation constant (M)	NIP apparent dissociation constant (M)
None	10.13×10^{-7}	4.69×10^{-4}
Porphyrin	5.30×10^{-7}	4.70×10^{-4}
Fe-porphyrin	3.40×10^{-7}	2.57×10^{-4}

According to the table, both porphyrin modifications increased the haemoglobin affinity in comparison with non-modified hydrogel. However, despite improvement in haemoglobin affinity, albumin binding was not lowered adequately by the free base porphyrin, which suggests insufficient selectivity.

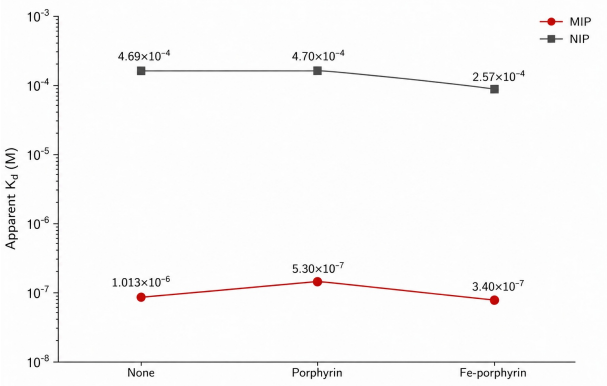

Figure 10: Apparent affinity.

As follows from the plot of affinity values, the imprinting effect manifests itself by providing lower dissociation constants of MIP compared with NIP. From the analytical perspective, this fact is important because of its effect on binding stability. Namely, weakly bound proteins can be washed away due to rinsing and/or washing-out processes [16–18].

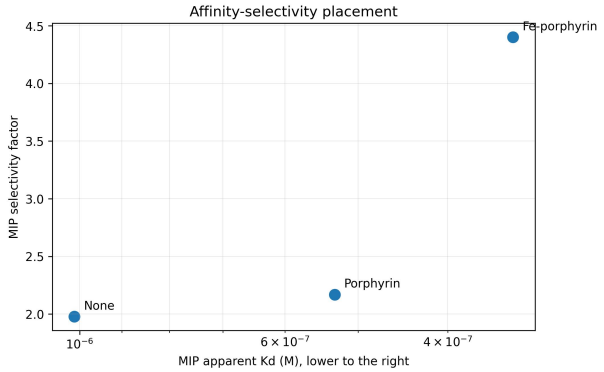

Figure 11: Affinity-selectivity plane.

Based on two criteria: high haemoglobin affinity and high selectivity to target vs interfering protein, the affinity-selectivity plane has been plotted. As can be seen from this plot, iron porphyrin imprint has the greatest advantages in these criteria, as it has the minimum dissociation constant and the maximum selectivity factor.

3.5. Interference-gated ranking

As can be seen from the table of contrasts derived based on experimental data, the imprint containing iron porphyrin provides the largest target interference window, the largest albumin rejection ratio, and the largest selectivity gain with respect to its non-imprinted version. Meanwhile, porphyrin shows relatively high imprinting gain but poor albumin rejection ratio.

Table 4: Derived analytical contrast variables.

Co-monomer	Target window (percentage points)	Imprint gain (percentage points)	Albumin rejection (percentage points)	Selectivity amplification (MIP/NIP)	Affinity separation (NIP K_d/MIP K_d)
None	36.5	23.4	13.1	1.98	463
Porphyrin	41.7	31.3	7.8	2.05	887
Fe-porphyrin	62.7	34.2	28.0	4.36	756

It can be said that the most optimal solution to the research problem is found. Namely, the free base porphyrin imprint demonstrates the highest affinity-separation measure; however, it has the lowest albumin rejection measure. Therefore, the imprint containing iron porphyrin wins the competition because of better overall balance in its analytical performance.

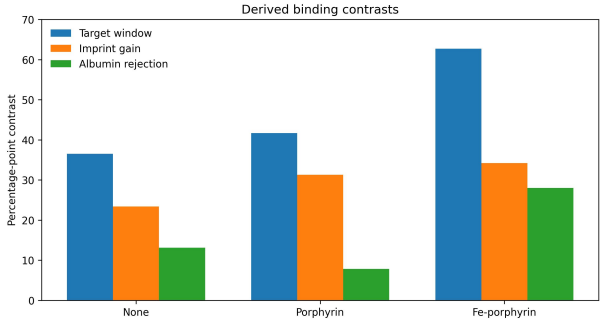

Figure 12: Binding contrasts.

As follows from the grouped contrast plot, free base porphyrin increases the imprint gain but provides relatively poor albumin rejection. On the contrary, iron-porphyrin imprint not only enhances the gain of the imprinting effect, but it also provides the largest differences between target and interference, as well as in albumin rejection ratio. Thus, metalation improves the material's interference blocking mechanism.

3.6. Mechanistic interpretation and analytical significance

The most probable reason for the observed effect may be cooperative recognition involving template-induced spatial configuration and chemistry of metalloporphyrin. In particular, it may be possible to use the combination of the former (cavity) with the latter (metal ion) to improve haemoglobin interaction with the material. The non-metallic porphyrin allows increasing binding through aromaticity and non-covalent interactions, but albumin adsorption is inevitable without metal ions.

Thus, the combination of metal ions and spatial configuration induced by the template seems to produce the effect. Indeed, even iron-porphyrin NIP has relatively high binding affinity to albumin, reaching 46.5 %. Only when it is combined with imprinting does the material begin to reject albumin successfully.

Therefore, it may be possible to conclude that metalation together with template induces selective environment, which is favorable to haemoglobin interactions, whereas albumin is rejected due to interference mechanisms.

This finding becomes extremely important in terms of instrument selection. Surface plasmon resonance chips, quartz crystal microbalance crystals, electrochemical electrodes, and optical probes are unable to distinguish target recognition from background fouling. Thus, the material with better affinity and lower interference is needed for reliable protein detection. Based on this analysis, only iron-porphyrin imprinting hydrogel is able to provide these properties.

4. Conclusion

Metalloporphyrin does provide good albumin blocking capabilities as it allows to reduce their interference to the minimum. The imprinting hydrogel containing iron porphyrin is the only one meeting the criteria of adequate resistance to interference defined by four parameters: haemoglobin binding ¿ 75 %, albumin binding ¡ 25 %, MIP selectivity factor ¿ 3, and MIP dissociation constant ¡ 5.0×10^{-7} M. The hydrogel with iron porphyrin meets all the requirements: haemoglobin binding 81.2 %, albumin binding 18.5 %, selectivity factor 4.40, dissociation constant 3.40×10^{-7} M.

As it can be seen, the effect is not associated with increased binding efficiency of hydrogels. Both free base and metal porphyrin do increase apparent affinity and bind more haemoglobin. However, free base does not lower albumin binding sufficiently to meet adequacy criterion. That is why the best choice is the metal porphyrin because of its reconfiguration of analyte separation: difference between haemoglobin–albumin binding decreased to 62.7 %points and albumin rejection to non-imprinted control increased to 28 %points.

Therefore, introduction of metal-centered porphyrin functionality into hydrogels is beneficial if the material is functionalized by template shaping its cavities. Thus, the most logical next step is to conduct further transducer-level validation of the material.

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